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Chlorophyll Regulates Accumulation of the Plastid-Encoded Chlorophyll Proteins P700 and D1 by Increasing Apoprotein Stability[1]

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Chlorophyll protein accumulation in barley (*Hordeum vulgare* L.) chloroplasts is controlled posttranscriptionally by light-induced formation of chlorophyll *a*. The abundance of translation initiation complexes associated with *psbA*, *psaA*, and *rbcL* mRNAs was measured using extension and inhibition analysis in plants grown in the dark for 4.5 d and then illuminated for up to 16 h. Light-induced accumulation of the chlorophyll proteins was not accompanied by changes in the abundance of translation initiation complexes, indicating that regulation of chlorophyll protein accumulation at this stage of development does not occur at the level of translation initiation. Translational runoff assays were performed in the presence of lincomycin, an inhibitor of translation initiation, to determine whether chlorophyll protein accumulation was regulated at the level of translation elongation. The extent of ribosome runoff of *psaA* and *psbA* mRNAs was similar in the presence or absence of chlorophyll, indicating that chlorophyll did not alter chlorophyll protein translation elongation. Polysome-associated D1 translation intermediates were radiolabeled in the presence or absence of chlorophyll, even though full-length D1 accumulated only in the presence of chlorophyll. Chlorophyll influenced the stability of D1 translation intermediates to a small extent and greatly increased D1 stability after release from ribosomes. Overall, these results demonstrate that light-induced chlorophyll biosynthesis triggers the accumulation of the chlorophyll proteins D1 and P700 in barley chloroplasts by enhancement of chlorophyll apoprotein stability.

The chloroplast thylakoid membrane contains six plastid-encoded Chl proteins: P700A, P700B, D1, D2, CP43, and CP47 (Shinozaki et al., 1986; Mullet, 1988). These Chl proteins mediate light-dependent primary charge separation and perform a light-harvesting antenna function in PSI and PSII. The P700 Chl apoproteins are encoded by *psaA* and *psaB*, respectively. These two subunits exhibit sequence similarity and constitute the transmembrane reaction center core in PSI (Almog et al., 1992). The *psaA* and *psaB* gene products contain nine helices each and together bind 45 Chl *a* molecules and β-carotene, phylloquinone, and an Fe-S center (Krauss et al., 1993). D1 and D2 are encoded by *psbA* and *psbD*, respectively. These proteins dimerize to form the photochemical reaction center core of PSII (Nanba and Satoh, 1987). D1 and D2 are homologous (Trebst, 1986) and are predicted to contain five transmembrane α helices based on structural similarities between bacterial reaction center proteins L/M and D1/D2 (Deisenhofer et al., 1985; Allen et al., 1987; Yeates et al., 1987; Deisenhofer and Michel, 1989; Feher at al., 1989) and immunological data (Sayer et al., 1986). The D1 and D2 heterodimer contains P680, a Chl *a* dimer, at least two additional antenna Chl molecules, quinones, pheophytins, and Fe^{2+}. CP43 and CP47, the antenna Chl proteins associated with PSII, are encoded by *psbC* and *psbB*, respectively. PSI and PSII contain other non-Chl-binding proteins that are encoded by either nuclear or plastid genes (Chitnis and Nelson, 1991; Vermas and Ikeuchi, 1991).

In monocots such as barley (*Hordeum vulgare* L.), the initial events of leaf and chloroplast development can occur in darkness, including leaf growth; increases in the number of plastids per cell, plastid DNA copy number, and transcription activity; and accumulation of plastid mRNAs and most chloroplast proteins (Robertson and Laetsch, 1974; Klein and Mullet, 1986, 1987; Mullet and Klein, 1987; Mullet, 1988; Baumgartner et al., 1989). However, Chl and the Chl proteins do not accumulate in dark-grown monocots. Illumination of dark-grown seedlings triggers the accumulation of Chl *a* and the Chl proteins (Vierling and Alberte, 1983; Klein and Mullet, 1986). Several lines of evidence indicate that accumulation of Chl *a* is the primary determinant of Chl protein accumulation. For example, the time course of light-induced Chl protein accumulation is similar to the time course of photoconversion of Pchlide to Chlide with subsequent formation of Chl *a* (Castelfranco and Beale, 1981; Vierling and Alberte, 1983; Klein and Mullet, 1986). Analysis of a Chl *a*-deficient barley mutant showed that light-induced conversion of Pchlide to Chl *a* is necessary for accumulation of Chl *a* apoproteins (Klein et al., 1988a). Furthermore, de novo synthesis of Chl *a* in isolated barley etioplasts is necessary and sufficient to trigger accumulation of the P700 apoproteins, CP43, CP47, and D1 (Eichacker et al., 1990).

Approximately equal amounts of Chl protein mRNAs are associated with polysomes in plastids isolated from dark-

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Abbreviations: CP43 and CP47, PSII chlorophyll-binding antenna proteins; D1 and D2, PSII chlorophyll-binding reaction center proteins; P700A and P700B, PSI chlorophyll-binding reaction center proteins; pD1, 34-kD precursor of D1.

grown and illuminated 4.5-d-old barley seedlings (Klein et al., 1988b). Based on these results and pulse labeling-chase experiments, it was suggested that light-mediated Chl synthesis stabilizes nascent Chl apoproteins, which results in their accumulation (Mullet et al., 1990). It has been reported that Chl-dependent accumulation of Chl proteins may also involve regulation of translation initiation and elongation (Eichacker et al., 1992).

In this study, toeprinting assays were used to measure the abundance of translation initiation complexes associated with *psbA*, *psaA*, and *rbcL* mRNAs in dark-grown and illuminated barley plants. This analysis revealed that accumulation of D1 and P700, triggered by Chl synthesis, was not accompanied by a change in the rate of translation initiation. Analysis of polysome runoff in the presence of an inhibitor of translation initiation showed that Chl has little influence on Chl protein translation elongation. This result was confirmed by analysis of pulse-labeled polysome-associated D1 translation intermediates. We conclude that Chl-induced accumulation of the chloroplast-encoded Chl proteins is mediated primarily by increased Chl apoprotein stability.

MATERIALS AND METHODS

Plant Growth and Plastid Isolation

Barley (*Hordeum vulgare* L. var Morex) seedlings were grown for 4.5 d in a dark chamber located in a light-tight room as described by Klein and Mullet (1987). After 4.5 d in the dark, seedlings were transferred to an illuminated chamber with a light intensity of 350 μE m^{-2} s^{-1} (incandescent plus fluorescent). All manipulations of dark-grown plants were performed when possible in complete darkness. However, when required, light was provided by a dim green safelight that was unable to photoconvert measurable amounts of Pchlide. Plastids were isolated from the apical 3 cm of the barley leaves on Percoll gradient as previously described (Klein and Mullet, 1987). For polysome isolation, chloramphenicol was included in grinding media and Percoll gradients at the concentration of 100 μg mL^{-1}.

Preparation of Polysomes for Toeprinting Analysis and Immunoprecipitations

Plastids isolated from barley seedlings grown under various conditions were lysed and fractionated into membrane and soluble phases as described by Klein and Mullet (1987). Membranes were resuspended in solubilization buffer (2% [w/v] polyoxyethylene 10-tridecylether, 50 mM Tris-HCl [pH 8.0], 25 mM KCl, 10 mM $MgCl_2$, 10 mM DTT). Insoluble materials were removed by microfuging samples for 5 min. Samples were loaded on a 2-mL RNase-free 33% (w/v) Suc cushion and centrifuged at 228,000*g* for 4 h in a TLA-100.3 rotor on a Beckman tabletop ultracentrifuge. Some polysome pellets were resuspended in toeprint assay buffer for extension and inhibition analysis (Hartz et al., 1988). When radiolabeled proteins were examined, proteins that remained above the Suc cushion and proteins that co-sedimented with polysomes were immunoprecipitated as previously described (Mullet et al., 1990). The antibodies used were formed against the C-terminal portion of D1 (amino acid No. 154–340).

Extension and Inhibition Reactions (Toeprinting)

Toeprinting reactions were done essentially as described by Hartz et al. (1988) and Kim et al. (1991) with minor modifications. The oligonucleotides used in this assay are complementary to nucleotides 148 to 167 numbered relative to the *psbA* mRNA 5′ end and nucleotides 386 to 403 numbered relative to the *rbcL* mRNA transcription start site. For toeprinting of *psaA* mRNA, the oligonucleotide used was complementary to nucleotides to +70 through +90 numbered from A of the AUG initiation codon based on rice chloroplast *psa*A DNA sequence (Hiratsuka et al., 1989). Each synthetic probe (50 pmol) was 5′ end labeled with [γ-^{32}P]ATP in a 50-μL reaction using T4 polynucleotide kinase (BRL) under standard conditions. Annealing mixtures containing polysomes and 5′-end-labeled probes were prepared as previously described (Kim et al., 1991) and incubated for 10 min at 37°C. Extension and inhibition reactions were performed as described by Kim et al. (1991). After the reactions were complete, samples were adjusted to 2% SDS and 20 mM EDTA, then extracted with phenol/chloroform, precipitated with ethanol, and dried. The pellets were resuspended with 10 μL of sequencing dye, heated for 3 min in a boiling water bath, and then placed on ice. Each reaction (5 μL) was separated on 8% denaturing polyacrylamide gels and autoradiographed. Sequencing reactions for *psbA* mRNA and *rbcL* mRNA were performed under the conditions previously described (Kim et al., 1991) with RNA generated in vitro by T7 RNA polymerase. Phenol-extracted plastid RNA was used for sequencing of *psaA* mRNA.

Protein Synthesis and Lincomycin Treatment

ATP-driven protein synthesis by intact plastids was performed as described by Klein and Mullet (1986) with various concentrations of lincomycin. Intact plastids were added at a final concentration of 1.375×10^7 plastids in a 75-μL reaction mixture containing [^{35}S]Met and incubated at 26°C. Reactions were terminated by adding 2.5 μL of unlabeled 5 mM Met and quickly freezing samples in liquid N_2. After the samples were thawed, radiolabeled plastids were fractionated into soluble and membrane phases (Klein and Mullet, 1987), solubilized in SDS, and electrophoresed on 7.5 to 15% polyacrylamide gels containing 4 M urea. Gels were fixed and fluorographed (Klein and Mullet, 1986). Radioactivity in bands corresponding to the Chl apoproteins was determined using a Betagen Betascope blot analyzer. Total radiolabel incorporation into membrane polypeptides was determined after TCA precipitation as described before (Mullet et al., 1986).

Translational Runoff Assay Using Intact Etioplasts

Previously described conditions for protein synthesis and Chl *a* synthesis in etioplasts were used except that 0.33 M sorbitol was included to keep plastids intact (Eichacker et al., 1990). Protein synthesis mixtures contained 1.6×10^8 etioplasts isolated from 4.5-d-old dark-grown plants. Plastids were incubated for 60 min at 26°C in the dark in the presence or absence of lincomycin (15.6 μM) and chloramphenicol (500 μg mL^{-1}). Unlabeled L-Met (0.1 mM) was included in the

reaction mixtures instead of [^{35}S]Met. Synthesis of Chl *a* in isolated intact etioplasts was achieved by a 15-s illumination of the translation assay mixtures that were supplemented with geranylgeranyl PPi (16 μM) and NADPH (0.25 mM). After the reactions were complete, plastids were fractionated into membrane and soluble phases. For the analysis of radiolabeled proteins, 1.375×10^7 plastids and [^{35}S]Met were added to protein synthesis mixtures, and plastids were incubated under the same conditions as described above. Membrane and soluble polypeptides were electrophoresed and fluorographed (Mullet et al., 1986).

Analysis of Plastid Polysomes on Suc Density Gradients

Plastids were fractionated into soluble and membrane phases (Klein et al., 1988b). The membrane fraction was solubilized in 700 μL of solubilization buffer (200 mM Tris-HCl [pH 8.5], 10 mM $MgCl_2$, 20 mM potassium acetate, 5 mM DTT, 0.5 mg mL^{-1} of heparin, 2% polyoxyethylene 10-tridecylether, and 1.5 mg mL^{-1} of proteinase K). Samples were kept on ice for 10 min and then microfuged for 5 min at 4°C. Samples were layered over linear gradients of 15 to 60% (w/v) RNase-free Suc in buffer containing 50 mM Tris-HCl (pH 8.5), 25 mM KCl, and 10 mM $MgCl_2$. Suc gradients were centrifuged at 288,000*g* for 2 h in a type SW41Ti rotor (Beckman Instruments Inc.). All operations were conducted at 4°C. After centrifugation, Suc gradients of polysomes were analyzed by pumping through a UV analyzer (model UA-5; Isco Inc., Lincoln, NE) with continuous monitoring of A_{254}.

RNA Isolation and Northern Analysis

mRNAs from intact plastids and Suc gradient fractions were isolated by phenol extraction as described by Mullet et al. (1985). Phenol-extracted tRNAs (Sigma, type XXI from *Escherichia coli*) were added to each extract at a final concentration of 10 μg mL^{-1}. Northern blot analyses were performed by separating RNA on 0.8% agarose gels containing 6% formaldehyde according to the Gene Screen instruction manual. RNA was transferred to Gene Screen membranes by capillary blot procedures and then UV cross-linked to the membranes with a UV cross-linker (Stratagene). Blots were prehybridized and then hybridized with in vitro-generated antisense RNA probes. RNA probes radiolabeled with [^{32}P]UTP were synthesized from recombinant pBluescript expression vectors with T7 or T3 RNA polymerase according to the supplier's instructions (Stratagene). Prehybridization, hybridization, and washing conditions were previously described for RNA probes (Sexton et al., 1990). The northern probe for *psbA* mRNA was a 1.3-kb *Hin*cII-*Eco*RI fragment from barley (Boyer and Mullet, 1988); the probe for *psaA* was a 1.0-kb *Bam*HI fragment from barley (Berends et al., 1987).

RESULTS

Analysis of *psbA, psaA,* and *rbcL* Translation Initiation Complexes

The toeprint assay, or extension and inhibition analysis, has been used previously to identify and localize translation initiation complexes on mRNA (Hartz et al., 1988). In this assay, a 5′-^{32}P end-labeled oligodeoxyribonucleotide complementary to a region downstream of a ribosome-binding site is annealed to mRNA. The hybridized primer is extended with reverse transcriptase, and ribosomes bound to the RNA cause chain termination. Analysis of radiolabeled cDNA termination products on a sequencing gel allows determination of the location of ribosomes bound to RNA. This assay has been used to determine the distribution of ribosomes on chloroplast polysomal mRNA and to identify ribosome-pausing sites correlated with paused translation intermediates (Kim et al., 1991).

If translation initiation is rate limiting, then changes in the rate of translation of a protein (at constant mRNA) should be paralleled by changes in the abundance of initiation complexes associated with specific mRNAs. Eichacker et al. (1992) suggested that light-induced accumulation of the plastid-encoded Chl apoproteins may result in part from increased rates of translation initiation. To test this idea, the abundance of translation initiation complexes associated with *psbA* and *psaA* in dark-grown and illuminated plants was analyzed.

Plastids were isolated from 4.5-d-old dark-grown plants and from 4.5-d-old dark-grown plants that had been illuminated for 0.25, 1, or 16 h. Plastids were fractionated into soluble and membrane phases, and the membrane phase was dissolved in a nonionic detergent. Polysomes were isolated by centrifuging the soluble phase and solubilized membrane materials separately through RNase-free Suc cushions. Each polysome pellet was subjected to toeprint analysis using probes that hybridize 60 to 70 nucleotides downstream from the AUG initiation codon of *psbA* (Fig. 1A), *rbcL* (Fig. 1B), or *psaA* (Fig. 1C). As shown in Figure 1A, the abundance of the initiation complex for *psbA* was high in etioplasts (lane 1, marked with an open arrow). Illumination of the 4.5-d-old dark-grown plants for up to 1 h did not increase the abundance of *psbA* translation initiation complexes, even though the rate of accumulation of radiolabel into D1 was markedly increased by this treatment (Klein and Mullet, 1986). After 16 h of illumination a slight increase in the abundance of the *psbA* initiation complex was observed (Fig. 1A, lane 4). The abundance of *rbcL* translation initiation complexes also remained constant upon illumination as shown in Figure 1B, which is consistent with little change in the rate of translation of large subunit of Rubisco during this treatment (Klein and Mullet, 1986, 1987).

The *psbA* translation initiation site lacks a Shine-Dalgarno sequence (GGAGG) often involved in ribosome binding in prokaryotes. In contrast, the *psaA* mRNA (encodes P700) contains a GGAGG sequence 4 to 12 nucleotides upstream of the initiator AUG. Therefore, translation initiation complexes associated with this region were analyzed. As shown in Figure 1C, the abundance of *psaA* translation initiation complexes was not changed when dark-grown barley plants were illuminated. A decrease of initiation complex abundance was observed after 16 h of illumination, which is paralleled by a corresponding decrease in polysome-associated *psaA* transcript level (Fig. 1C, estimated by the decrease in abundance in *psaA* transcript 5′ termini marked with arrow a). Phenol extraction of the polysomes prior to the toeprinting assay generated a stack of new strong bands located just below the toeprint signal generated by the *psaA* translation

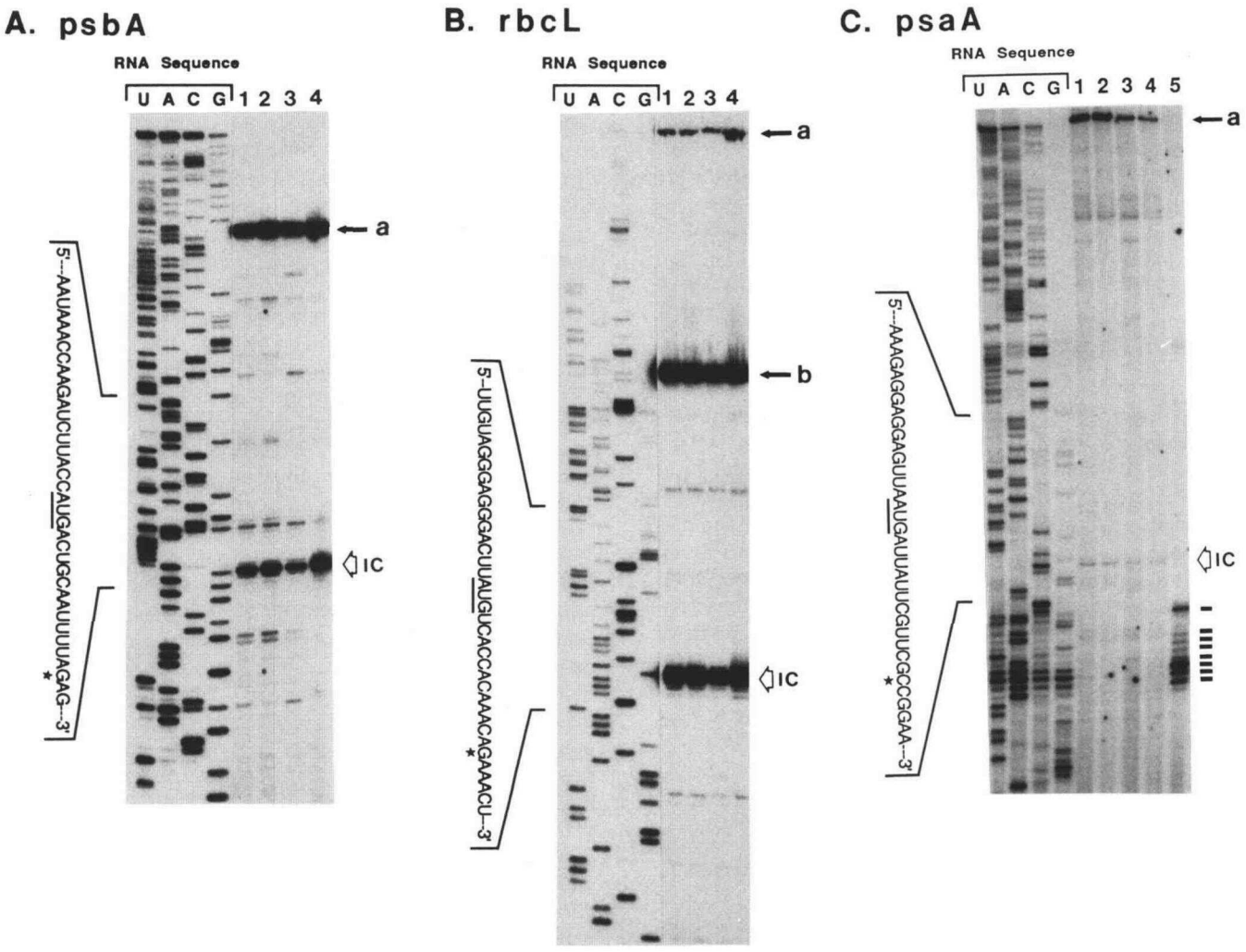

Figure 1. Toeprinting analysis of translation initiation complexes associated with *psbA* (A), *rbcL* (B), and *psaA* (C) mRNAs from dark-grown and illuminated seedlings. Intact plastids were isolated from seedlings grown for 4.5 d in the dark and then illuminated for 0 (lane 1), 0.25 (lane 2), 1 (lane 3), and 16 h (lane 4). Lane 5 of C is the toeprint of phenol-extracted polysomes. Arrows, a and b, indicate the 5′ ends of each transcript. The open arrow labeled as IC marks the position of the translation initiation complex on each transcript. Thick bars shown below the open arrow in C indicate signals induced by RNA secondary structures. RNA sequences were generated using in vitro-synthesized RNAs for *psbA* and *rbcL* and phenol-extracted plastid RNA for *psaA*. RNA sequences around translation initiation codons are drawn at the left of each figure. The AUG initiation codon is underlined, and toeprint signals induced by chloroplast ribosomes are marked with asterisks on the RNA sequence.

initiation complex (Fig. 1C, lane 5). Potential stem-loop structures were located in the *psaA* initiation site, which included the AUG initiation codon and GGAGG sequence (data not shown). Ribosome-binding or RNA-binding proteins may open up this secondary structure during translation initiation. The lack of change in the abundance of *psbA* and *psaA* translation initiation complexes upon illumination indicates that translation initiation is not involved in the light-induced accumulation of Chl apoproteins at this stage of plastid development. These results suggest that regulation occurs at the level of translation elongation and/or protein stability.

Translational Runoff Assay with Intact Etioplasts

Chl apoprotein accumulation might be activated when Chl binds co-translationally to nascent Chl apoproteins and releases a block in translation elongation (Klein et al., 1988b; Eichacker et al., 1992). Alternatively, Chl may bind to and stabilize the Chl apoproteins, which results in their accumulation. The latter mechanism predicts that the Chl apoproteins are being synthesized in etioplasts, but in the absence of Chl, rapid turnover occurs such that little full-length apoprotein can be detected. In the present study, experiments were designed to distinguish these two possible mechanisms using translational runoff assays with intact etioplasts. As diagrammed in Figure 2, if apoprotein degradation regulates the amount of Chl apoprotein accumulated, then mRNA should shift into smaller polysomes during translation runoff assays in the presence or absence of Chl *a*. If, however, translation elongation is blocked in the absence of Chl *a*, then less shift should be observed in the absence of Chl *a*. In the translation

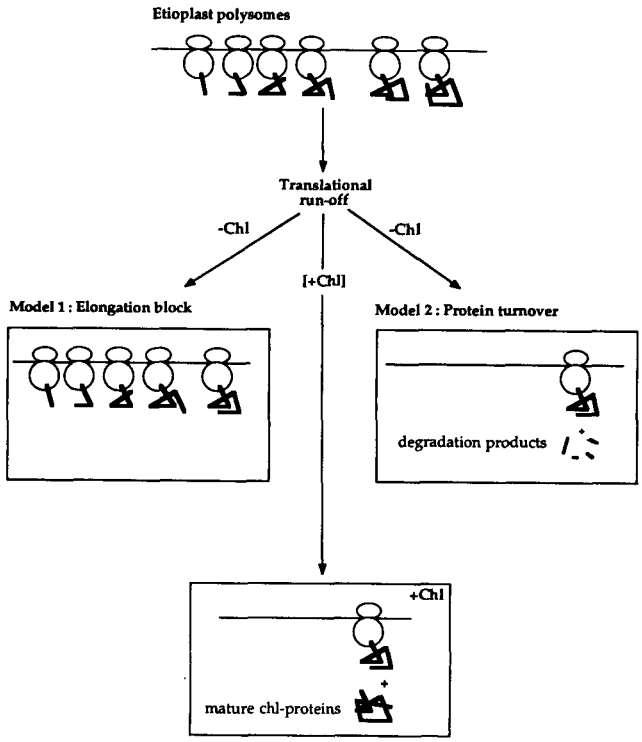

Figure 2. Diagram showing possible results of translational runoff assays in the presence or absence of Chl *a*. 30S and 50S subunits of ribosomes are drawn as ellipses and circles, respectively. Translation intermediates are drawn as broken thick bars. −Chl and +Chl denote the absence or presence of Chl *a*, respectively.

assay, Chl apoproteins would accumulate only in the presence of Chl *a*, but non-Chl-binding proteins (e.g. the large subunit of Rubisco) would accumulate in the presence and absence of Chl *a*.

Ribosome runoff in the presence or absence of Chl *a* synthesis was assayed by determining the distribution of polysomes on Suc density gradients. As shown in Figure 3, C and E, the readout of ribosomes was similar in both conditions. Since the changes in polysome profile could be due to the degradation of polysomal mRNA, chloramphenicol, an inhibitor of polypeptide chain elongation, was included in some experiments. As shown in Figure 3, B and D, overall polysome profiles were preserved after incubation of translation mixes in the presence of chloramphenicol, indicating that changes in polysome profiles in the absence of this inhibitor were due to ribosome runoff. Significant amounts of disomes, trisomes, and larger polysomes were present even after 60 min of translation. This was probably due to the reinitiation of translation during the assay and gradual inhibition of translation elongation in isolated plastids.

To prevent reinitiation during the runoff assay, a translation initiation inhibitor was included in the translational runoff assay. Two inhibitors of bacterial initiation, kasugamycin and lincomycin were tested for their ability to inhibit plastid translation initiation. Kasugamycin inhibits initiation in bacteria by preventing the binding of *N*-formyl-methionyl-tRNA to the 30S subunit, and in lysed plastids, kasugamycin inhibits plastid translation initiation (Eichacker et al., 1992). However, kasugamycin did not inhibit translation in intact plastids presumably because of the lack of transport across the plastid envelope. Lincomycin is known to inhibit transpeptidation soon after initiation, leading to the formation of short peptide chains. In addition, lincomycin inhibits the binding of P-site substrates to the peptidyltransferase center and has no effect on polysome runoff in bacterial cells. Lincomycin effectively inhibited plastid translation initiation in intact plastids (Fig. 4). At low concentrations of lincomycin

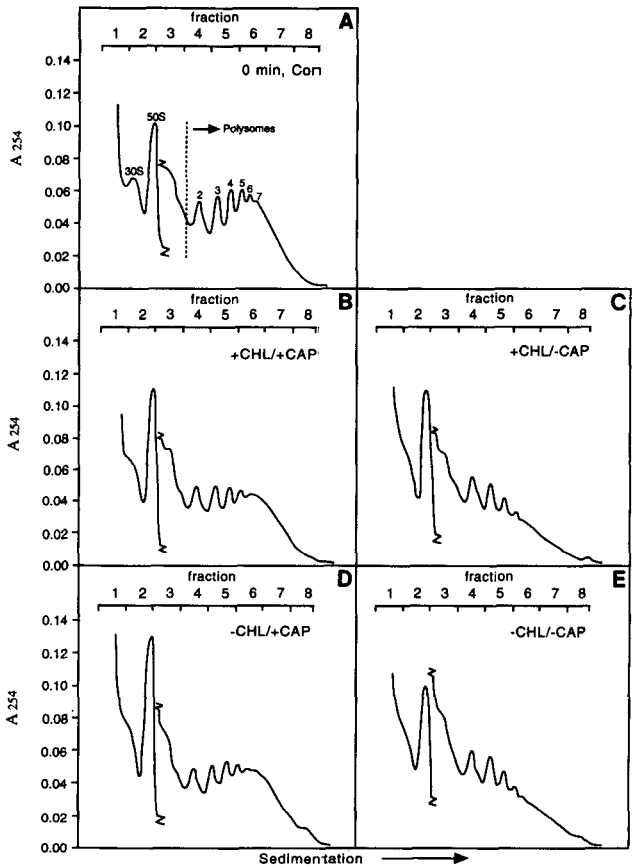

Figure 3. Suc density gradient profiles of membrane-associated ribosomal material of dark-grown plastids in which the ribosomes were allowed to run off under various conditions. Plastids (1.6×10^8) were isolated from 4.5-d-old dark-grown seedlings and incubated in translation mixture for 0 or 60 min at 26°C in the dark in the presence (B and C) or absence (D and E) of Chl *a* (CHL). Chl *a* was synthesized in isolated intact etioplasts as described in "Materials and Methods." Chloramphenicol (CAP) was included in some experiments during the incubation (B and D). After the reactions were complete, plastids were fractionated into membrane and soluble phases. Membranes were solubilized in nonionic detergent and solubilized membrane materials layered over 15 to 60% Suc gradients, centrifuged, and scanned at A_{254}. Gradients of ribosomal materials were fractionated into eight fractions as indicated. A 2-fold decrease in full-scale *A* is indicated by broken bars in the *A* tracing of each profile. The top of each gradient is to the left. The numbers above the polysome peaks in A denote the number of the ribosomes associated with RNA.

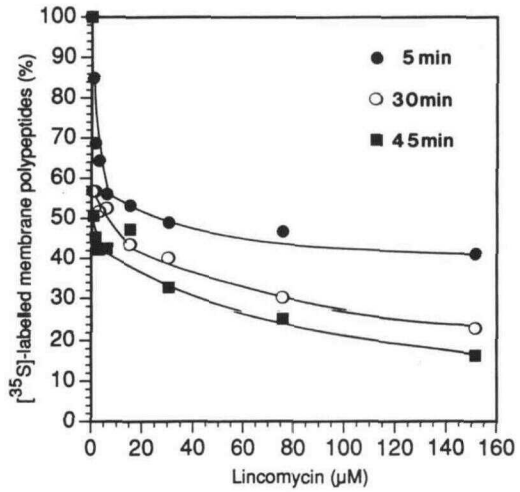

Figure 4. The effect of various concentrations of lincomycin on the incorporation of [^{35}S]Met into plastid membrane polypeptides. Plastids were isolated from seedlings grown in the dark for 4.5 d and then illuminated for 1 h. Intact plastids were incubated in translation mixtures containing [^{35}S]Met at 26°C for 5 (●), 30 (○), and 45 min (■). After the reactions were terminated, plastids were fractionated into membrane and soluble phases. The radiolabeled membrane polypeptides were counted by measuring the TCA-insoluble radioactivity as described by Mullet et al. (1986).

(3 μM), the incorporation of [^{35}S]Met into membrane polypeptides was reduced 40%. Further increases in inhibitor concentration increased inhibition somewhat, probably through secondary inhibition of elongation. Further evidence that lincomycin inhibits initiation was obtained by examining the time course of protein synthesis in the presence or absence of lincomycin (Fig. 5). Since, in the first 5 or 10 min, label is incorporated primarily into proteins that were initiated in vivo, the influence of lincomycin is expected to be greater at later times of translation. As shown in Figure 5, lincomycin inhibited radiolabel incorporation into plastid proteins less during the first 5 to 10 min compared to later times, consistent with inhibition of translation initiation.

Translational Runoff Assays in the Presence or Absence of Chl *a*

Figure 6 shows the profiles of membrane-bound polysomes and the corresponding distribution of *psaA-psaB* and *psbA* mRNA in the polysome gradients. Northern analysis of *psaA* mRNA distribution in polysome profiles showed that *psaA* mRNA shifted into smaller polysomes during translation in the presence or absence of Chl *a*. Similar results were observed for *psbA* mRNA, although the change in mRNA distribution was less dramatic than for *psaA* mRNA. In the presence of chloramphenicol, little change in mRNA distribution was observed, indicating that mRNA degradation was not causing the observed changes in mRNA distribution in the polysome fractions. These results indicate that translation elongation of the Chl apoproteins is not significantly influenced by Chl.

In organello translation assays were performed under the same conditions used for the polysome runoff assay shown in Figure 6. In the absence of Chl *a* synthesis, P700 apoproteins did not accumulate in the presence or absence of lincomycin (Fig. 7, lanes 1 and 3). In contrast, synthesis of Chl *a* during translation resulted in a large accumulation of P700 apoproteins (Fig. 7, lanes 2 and 4). D1, CP43, and CP47 accumulated to a greater extent when translations were carried out in the presence of Chl. In contrast, accumulation of D2 and the non-Chl-binding large subunit of Rubisco was not effected by Chl as previously reported (Klein and Mullet, 1986, 1987). Although lincomycin reduced overall incorporation, this inhibitor had little effect on the protein profiles or the influence of Chl on protein accumulation.

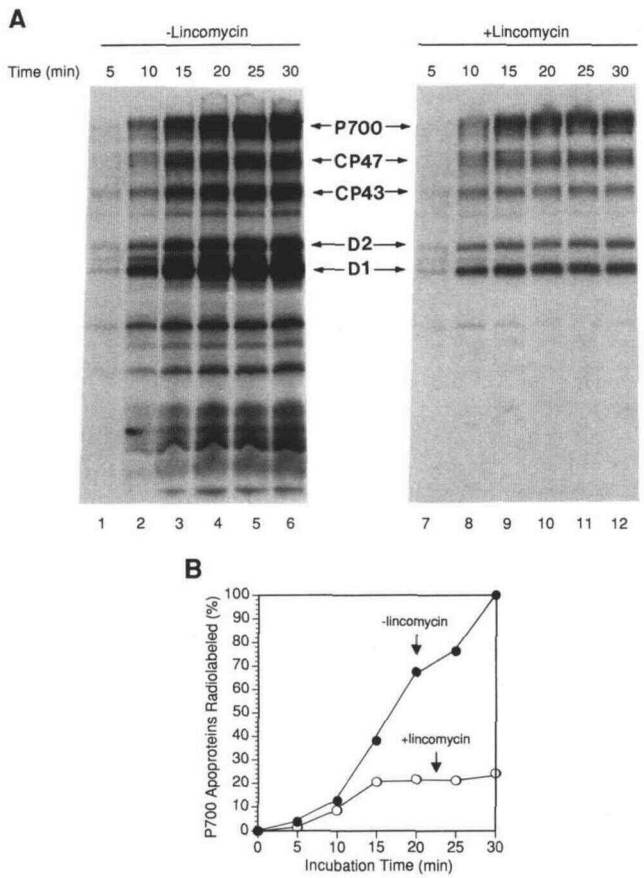

Figure 5. Synthesis of plastid membrane polypeptides as a function of incubation time in the presence or absence of lincomycin. Intact plastids were isolated from seedlings grown in the dark for 4.5 d and illuminated for 1 h and incubated with [^{35}S]Met at 26°C for 5, 10, 15, 20, 25, and 30 min in translation mixture with (lanes 7–12) or without (lanes 1–6) lincomycin (15.1 μM). After the reactions were complete, radiolabeled plastids were fractionated into membrane and soluble phases, and membrane samples were electrophoresed, fluorographed, and autoradiographed (A). The radiolabel incorporated into P700 was quantitated with a Betagen Betascope blot analyzer and plotted as a function of time (B). P700, Chl apoproteins A and B of PSI; CP47, 47-kD PSII Chl apoprotein; CP43, 43-kD PSII Chl apoprotein; D2, 34-kD PSII reaction center polypeptide; D1, 32-kD PSII reaction center polypeptide.

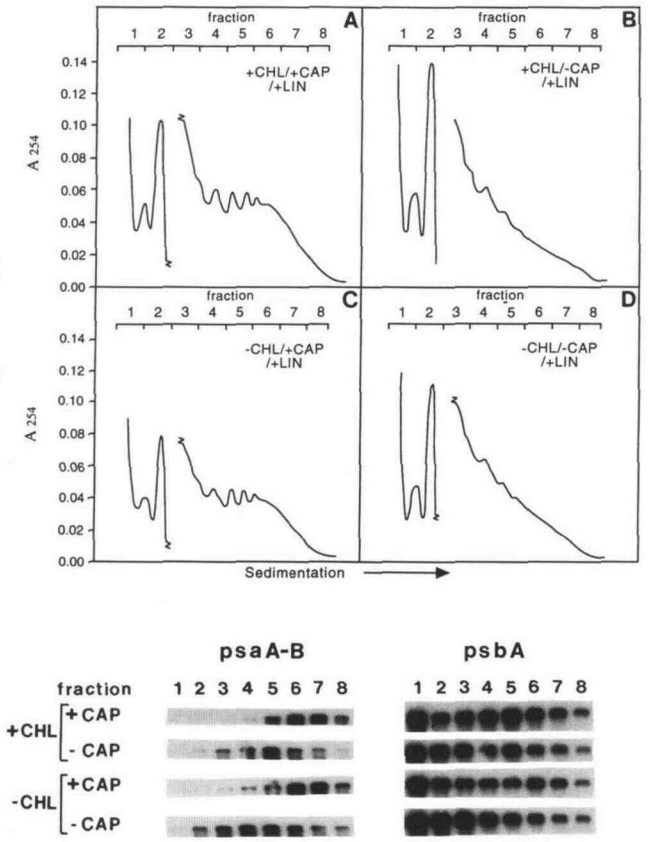

Figure 6. Suc density gradient profiles of membrane-associated ribosomal material of dark-grown plastids in which the ribosomes were to run off in the presence of lincomycin under various conditions (A–D). Northern blot analyses of RNA from Suc density gradients are shown in the lower part of the figure. Plastids (1.6×10^8) were isolated from 4.5-d-old dark-grown barley seedlings and incubated for 60 min at 26°C in the dark in the presence (1 and 2) or absence (3 and 4) of Chl *a* (CHL). Chl *a* was synthesized in isolated intact etioplasts as described in "Materials and Methods." Lincomycin was included at 15.1 μM in the reaction mixture. Chloramphenicol (CAP) was included in some experiments during the incubation (A and C). After the reactions were complete, plastids were fractionated into membrane and soluble phases. Solubilized membrane materials were layered over 15 to 60% Suc gradients, centrifuged, and scanned at A_{254}. Gradients of ribosomal materials were fractionated into eight fractions as indicated. A 2-fold decrease in full-scale *A* is indicated by broken bars in the *A* tracing of each profile. Nucleic acid from Suc gradient fractions was isolated by phenol extractions. Nucleic acid of each fraction was loaded on 6% formaldehyde gels and subsequently electrophoresed, transferred to nylon membranes, and hybridized with antisense RNA probes for *psaA* and *psbA* (bottom).

Polysome-Associated D1 Translation Intermediates Accumulate in the Absence of Chl

The experiments described above indicate that Chl apoprotein translation continues in the absence of Chl and that lack of Chl apoprotein accumulation under these conditions results from protein turnover. We next analyzed the influence of Chl on the stability of Chl protein nascent chains associated with polysomes. Plastids from dark-grown plants were isolated and pulse labeled with or without concurrent Chl biosynthesis. After the pulse-labeling treatment, plastid membranes were isolated, and proteins associated with polysomes and polysome-free proteins were separated on Suc gradients, followed by immunoprecipitation with antibodies against the C terminus of D1. The results of this experiment are shown in Figure 8. As expected, polysome-free pD1 and D1 accumulated in the membrane phase when translation was carried out in the presence of Chl but not in the absence of Chl (Fig. 8A, lanes 7 and 8 versus lanes 1 and 2). When plastids were radiolabeled in the absence of Chl and then chased in the presence of Chl, a small amount of pD1 accumulated (Fig. 8A, lane 6). D1 accumulation did not occur if the chase was carried out in the absence of Chl or if chloramphenicol was added prior to the chase to prevent ribosome readout (Fig. 8A, lanes 3, 4, and 5). When plastids were radiolabeled in the presence of Chl and then chased in the absence of chloramphenicol, additional label accumulated

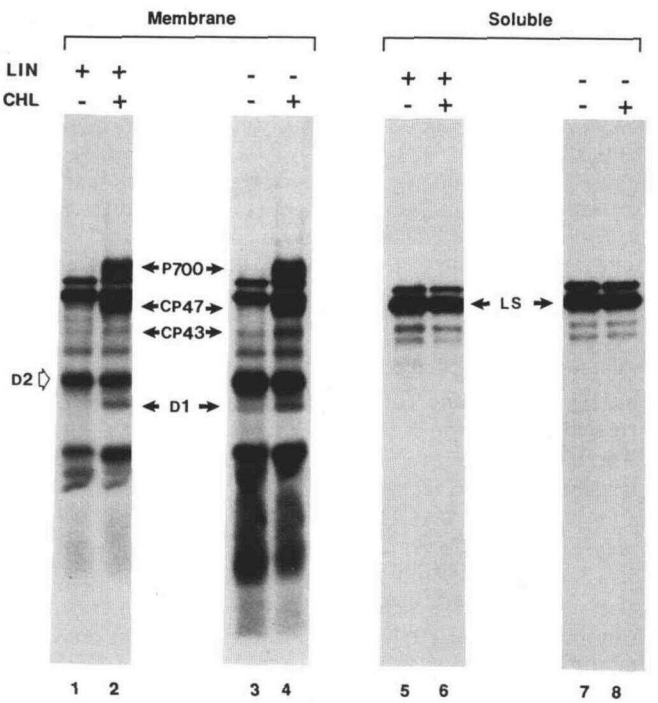

Figure 7. The effect of Chl *a* on accumulation of radiolabeled membrane and soluble polypeptides by plastids isolated from dark-grown seedlings. Intact plastids were isolated from 4.5-d-old dark-grown seedlings and incubated with [^{35}S]Met for 60 min in translation mixtures in the presence or absence of Chl *a* (CHL) and lincomycin (LIN). Incubation conditions were the same as described in the legends to Figures 6 and 7 except that a smaller number of plastids (1.4×10^7) were used and [^{35}S]Met was included for radiolabeling the plastid polypeptides. After the reactions were complete, radiolabeled plastids were fractionated into membrane and soluble phases, and proteins were electrophoresed, fluorographed, and exposed to Kodak x-ray film for approximately 26 h (lanes 3, 4, 7, and 8) or 62 h (lanes 1, 2, 5, and 6). P700, P700 Chl apoproteins A and B of PSI; CP47, 47-kD PSII Chl apoprotein; CP43, 43-kDa PSII Chl apoprotein; D2, 34-kD PSII reaction center polypeptide; D1, 32-kD PSII reaction center polypeptide; LS, large subunits of Rubisco.

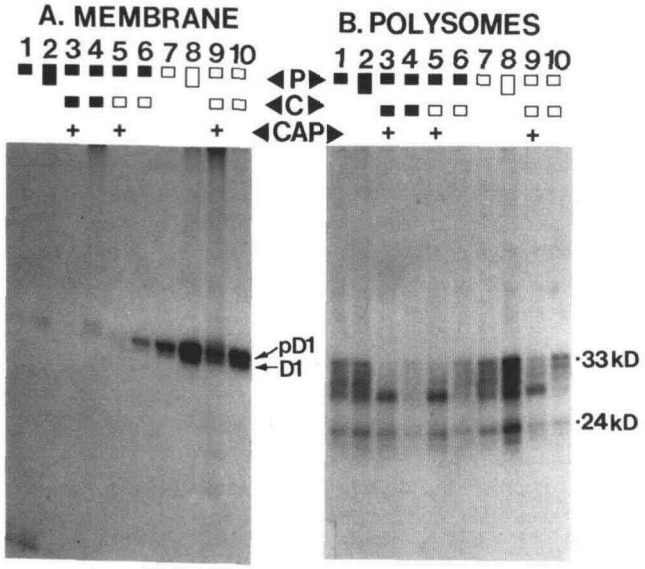

Figure 8. Analysis of D1 and D1 translation intermediates pulse labeled in the presence or absence of Chl. Plastids were pulse labeled (top row of boxes labeled P) with [^{35}S]Met for 10 min (lanes 1, 3–7, 9, and 10) or for 20 min (lanes 2 and 8) in the presence (open boxes) or absence (solid boxes) of Chl. Chases (second row of boxes labeled C) were carried out for 10 min in the presence (open boxes) or absence (solid boxes) of Chl. In some cases, chloramphenicol (CAP) was added prior to the chase (lanes marked with +). Membrane-bound, non-polysome-associated pD1 and D1 are labeled in A. Polysome-associated D1 translation intermediates are shown in B. The approximate molecular masses of some the D1 translation intermediates are shown at the right of the figure.

in D1 (Fig. 8A, lane 10). This results from readout of radiolabeled translation intermediates during the chase.

In a previous study, we used an antibody against the N terminus of D1 to identify D1-paused translation intermediates of 15 to 18, 21, 24, and 28 to 33 kD associated with polysomes (Kim et al., 1991). In the present study, translations were carried out in the presence or absence of Chl, and after isolation of polysomes, D1 translation intermediates were immunoprecipitated with antibodies against the C terminus of D1 (Fig. 8B). Plastids pulse labeled for 10 min in the presence or absence of Chl revealed D1 translation intermediates of approximately 24 to 33 kD associated with polysomes (Fig. 8B, lanes 1 and 7). The regular spacing between bands in the 28 to 33 kD range may indicate that termination and release of full-length pD1 is rate limiting and polysomes stack up at the termination codon. More important is the observation that the abundance of these translation intermediates in a 10-min translation assay was influenced to only a small extent by Chl (Fig. 8B, lane 1 versus 7). After 20 min of translation, the abundance of D1 translation intermediates was increased in the presence of Chl (Fig. 8B, lane 2 versus 8). This difference after longer periods of translation may be related to Chl-induced membrane association and stabilization of the D1 translation intermediates. The stability of the D1 translation intermediates was examined by adding chloramphenicol after the chase and allowing further incubation for 10 min (Fig. 8B, lanes 3, 5, and 9). This treatment resulted in the loss of D1 translation intermediates between 29 and 33 kD with greater turnover occurring in the absence of Chl (Fig. 8B, compare lane 3 with 9). If chases were carried out in the presence of unlabeled Met, radiolabel in the D1 translation intermediates decreased as a consequence of ribosome readout (Fig. 8B, lanes 4, 6, and 10).

DISCUSSION

Light modulates several steps in leaf and chloroplast development in plants. In particular, light-induced Chl biosynthesis triggers the accumulation of the chloroplast-encoded Chl proteins (Klein et al., 1988a; Eichacker et al., 1992). Chl does not alter Chl apoprotein mRNA level or the distribution of mRNA in polysomes, indicating that Chl activates Chl protein accumulation posttranscriptionally (Klein et al., 1988b). In this paper, toeprint assays were used to determine whether the Chl-induced accumulation of the Chl proteins was accompanied by a change in translation initiation. Analysis of *psbA*, *rbcL*, and *psaA* translation initiation regions revealed the presence of translation initiation complexes in etioplasts of 4.5-d-old dark-grown barley seedlings. The abundance of the initiation complexes on these mRNAs changed very little when plants were illuminated for up to 1 h. In contrast, accumulation of radiolabel into the Chl apoproteins is minimal in etioplasts and reaches a maximum within 1 h of illumination (Klein and Mullet, 1986). Therefore, we conclude that Chl-induced accumulation of the Chl proteins is not mediated by activation of translation initiation at this stage of chloroplast development. This conclusion is consistent with findings from previous studies showing that light-induced accumulation of Chl and the Chl proteins was not accompanied by a significant or selective shift of the Chl protein mRNAs in polysome profiles (Klein et al., 1988b).

Chl-induced accumulation of the Chl proteins might be mediated through activation of translation elongation (Eichacker et al., 1992). We have shown previously that ribosomes pause during translation of D1 (Kim et al., 1991). Therefore, it was possible that translation elongation is a rate-limiting and regulated step in Chl protein synthesis. This possibility was analyzed by determining whether Chl altered the readout of ribosomes bound to *psbA* and *psaA* mRNAs. This analysis was improved by the use of lincomycin, which blocks translation initiation. The translation readout assays showed that Chl did not influence overall translation elongation on *psaA* and *psbA* mRNAs. The lack of a direct effect of Chl on translation elongation is consistent with quantitation of ribosome pausing in the presence and absence of Chl (J. Kim and J.E. Mullet, unpublished data).

Previous attempts to pulse label D1 in the absence of Chl were unsuccessful, presumably because under these conditions D1 is sufficiently unstable to preclude significant buildup of the Chl apoprotein (Mullet et al., 1990). Therefore, it has not been possible to directly measure the half-life of D1 in the absence of Chl. In this manuscript we addressed this question indirectly by pulse labeling D1 translation intermediates associated with polysomes with or without concurrent Chl biosynthesis. D1 translation intermediates associated with polysomes were identified by immunoprecipitation with antibodies against D1. This experiment showed

that D1 translation was occurring in the presence and absence of Chl and that in 10-min pulse-labeling assays, Chl had only a small influence on the abundance of D1 translation intermediates. When the stability of the translation intermediates was determined by pulse-chase assays in the presence of chloramphenicol, Chl was found to increase the stability of the 28- to 33-kD translation intermediates to a small extent. We interpret these results to indicate that Chl influences the stability of D1 to a small extent during translation and to a much larger extent after D1 is released from the ribosome.

Free Chl is a potent mediator of membrane photooxidation. Chl-mediated photooxidation is prevented by binding Chl in Chl proteins, which facilitate the transfer of excitation energy to reaction centers for photochemistry. In addition, the Chl proteins bind carotenoids, which quench Chl triplet states and help prevent the generation of oxygen radicals. Therefore, it is reasonable for plastids to synthesize Chl apoproteins in excess so that whenever Chl is synthesized a Chl apoprotein is always available.

The results in this paper lead us to conclude that Chl activates the accumulation of the chloroplast-encoded Chl proteins primarily by binding to and stabilizing the Chl apoproteins. Evidence for Chl-induced stabilization of CP43 was previously provided by pulse-chase assays (Mullet et al., 1990). Similarly, other Chl proteins including the nuclear-encoded Chl *a/b*-binding light-harvesting complexes associated with PSII apoproteins (Bennett, 1981), PSI Chl proteins in *Chlamydomonas* (Herrin et al., 1992), and bacteriochlorophyll-binding proteins of photosynthetic bacteria are stabilized by Chl (Dierstein, 1983).

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